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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of	: Yoshiharu MATAHIRA et al.
Serial No.	: 10/076,686
Filed	: February 14, 2002
For	: METHOD OF SKIN CARE
Art Unit	: 1614
Examiner	: Clinton Ostrup

DECLARATION UNDER 37 CFR 1.132

HONORABLE COMMISSIONER OF PATENTS AND TRADEMARKS

WASHINGTON, D.C. 20231

SIR;

Now comes Yoshiharu MATAHIRA who deposes and says that:

1. I am a co-inventor of the invention described and claimed in the above-referenced application.

2. I graduated from Shizuoka University, Faculty of Agriculture, Department of Agricultural Chemistry in 1984, and received my doctoral degree in agriculture from Gifu University, United Graduate School, Agricultural Research Course in 1995, and has been employed by Yaizu Suisan Kagaku Industry Co., Ltd. since 1984.

3. Under my supervision and control, the following experiments were carried out:

TEST EXAMPLE **A** (Influential test of NAG and collagen on skin)

30 females usually having troubles of chronic xeroderma and rough skin were invited for the following test:

Background of females to be tested:

	NAG+C group	NAG group	C group
Number of symptomatic persons	10	10	10
Average age	30.5	29.8	30.7
Average height (cm)	157.8	156.9	158.2
Average weight (kg)	52.8	52.6	53.4
Average sleeping hours (hr)	7.1	6.9	6.8

(NAG+C group: N-acetylglucosame and collagen were applied, NAG group: N-acetylglucosame was applied, and C group: collagen was applied.)

Samples to be applied (gram per 50 ml drink)

	NAG+C group	NAG group	C group
NAG	0.5	0.5	-
Collagen	2.5	-	2.5
High Fructose corn syrup	2.5	2.5	2.5
Sugar	2.5	2.5	2.5
Vitamin C	0.25	0.25	0.25
Citric acid	0.1	0.1	0.1
Perfume	0.05	0.05	0.05
Preservative	0.01	0.01	0.01

The above materials were dissolved in water, and made up to 50 ml.

Test methods:

Each of the symptomatic persons was asked to drink the sample to be applied (50 ml of drink) per day before breakfast. The period of ingestion (administration) was 8 weeks for each person. Inspection was carried out before ingestion, after 4 weeks and after 8 weeks.

Methods of inspection:

(1) Dermatologic examination and doctors questions (Face and whole body)

(2) Moisture content, oil and fat content, and acidity (pH)

(3) Analysis by microscopic three-dimensional skin surface

TEST RESULTS:

(1) Dermatologic examination and doctors' questions

Inspection was carried out in accordance with the TEST EXAMPLE 5, describing on page 16, lines 25 continued over page 17 line 7 in the specification of the above-identified application.

Table 1

		NAG-C group (n=10)			
		Number of symptomatic persons	Before ingestion	After 4 weeks	After 8 weeks
Face	Cosmetic dermatitis	5	1.18	1.09	1.13
	Desiccation	9	2.08	1.15*	1.02*
	Flushing	8	1.87	1.06*	1.08*
	Spread of cosmetics	9	1.83	1.03*	0.77**
Whole body	Prutitis	9	1.87	0.78*	0.71**
	Desiccation	10	2.09	1.36*	1.03**
	Flushing	5	0.98	0.92	0.45
	Erosion	3	2.05	1.03	1.03
	Desquamation	6	2.07	1.23	0.87*
	Papula	3	1.35	1.57	1.55
	Vesicle	2	1.00	2.00	2.00
General observation		10	2.02	1.47*	1.12**

Wiscoxon test: *:$p<0.05$, **:$p<0.01$

Table 2

		NAG group (n=10)			
		Number of symptomatic persons	Before ingestion	After 4 weeks	After 8 weeks
Face	Cosmetic dermatitis	8	1.18	1.09	1.13
	Desiccation	10	2.27	1.89	1.32*
	Flushing	5	1.65	1.32	1.60
	Spread of cosmetics	8	1.97	1.83	1.18*
Whole body	Prutitis	9	1.87	1.90	1.31
	Desiccation	10	1.83	1.66	1.33
	Flushing	6	1.69	1.54	0.76
	Erosion	1	2.00	1.00	1.00
	Desquamation	7	2.12	1.83	1.65
	Papula	2	1.67	1.51	1.45
	Vesicle	1	2.00	1.00	2.00
General observation		10	2.02	1.87	1.38*

Wiscoxon test: *:p<0.05, **:p<0.01

Table 3

		C group (n=10)			
		Number of symptomatic persons	Before ingestion	After 4 weeks	After 8 weeks
Face	Cosmetic dermatitis	10	1.45	1.31	1.33
	Desiccation	10	2.01	1.79	1.80
	Flushing	6	1.72	1.54	1.69
	Spread of cosmetics	8	2.13	1.88	1.39*
Whole body	Prutitis	10	1.57	1.73	1.39
	Desiccation	10	1.83	1.78	1.50
	Flushing	5	1.69	1.33	0.96
	Erosion	0	0.00	0.00	0.00
	Desquamation	6	1.92	1.63	1.69
	Papula	1	2.00	2.00	2.00
	Vesicle	1	2.00	2.00	1.00
General observation		10	2.07	1.99	1.98

Wiscoxon test: *:p<0.05, **:p<0.01

(2) Moisture content, oil and fat content, and acidity (pH)

Inspection was carried out in accordance with the TEST EXAMPLE 5, describing on page 18, line 10 continued over page 19, line 26 in the specification of the above-identified application.

Table 4

		NAG-C group (n=10)		
		Before ingestion	After 4 weeks	After 8 weeks
Moisture content	Below left eye	52.2±10.3	54.9±9.1	59.1±6.8*
	Left upper arm	38.5±8.9	41.5±7.6	47.2±6.9*
	Poll	50.8±9.5	52.7±10.3	57.6±12.6
Acidity (pH)	Below left eye	5.8±0.4	5.6±0.2	5.6±0.7
	Left upper arm	5.6±0.5	5.8±0.5	5.7±0.8
	Poll	5.7±0.3	5.5±0.4	5.6±0.6
Oil and fat content	Below left eye	38.21±20.2	32.1±13.6	38.6±15.1

Wiscoxon test: *:p<0.05, **:p<0.01

Table 5

		NAG group (n=10)		
		Before ingestion	After 4 weeks	After 8 weeks
Moisture content	Below left eye	51.7±12.3	52.7±10.1	55.1±8.2
	Left upper arm	40.5±6.7	42.3±8.4	42.1±7.3
	Poll	51.8±10.4	54.1±12.6	52.6±11.9
Acidity (pH)	Below left eye	5.5±0.3	5.8±0.2	5.6±0.8
	Left upper arm	5.5±0.6	5.7±0.8	5.7±1.1
	Poll	5.6±0.6	5.5±0.7	5.6±0.5
Oil and fat content	Below left eye	37.3±19.6	37.1±16.1	38.5±17.3

Wiscoxon test: *:p<0.05, **:p<0.01

Table 6

		C group (n=10)		
		Before ingestion	After 4 weeks	After 8 weeks
Moisture content	Below left eye	55.3±10.1	57.4±12.9	58.1±9.2
	Left upper arm	43.2±11.3	45.5±9.4	43.8±6.9
	Poll	50.2±11.8	52.0±11.3	52.2±10.4
Acidity (pH)	Below left eye	5.7±0.9	5.6±0.2	5.5±0.7
	Left upper arm	5.8±0.3	5.7±0.4	5.6±0.5
	Poll	5.7±0.4	5.5±0.5	5.6±0.3
Oil and fat content	Below left eye	36.2±13.9	37.3±15.9	38.0±18.3

Wiscoxon test: *:p<0.05, **:p<0.01

(3)Analysis by microscopic three-dimensional skin surface analyzer

Inspection was carried out in accordance with the TEST EXAMPLE 5, describing on page 21, line 3 continued over page 23, line 6 of the specification of the above-identified application.

Table 7

		NAG-C group (n=10)		
		Before ingestion	After 4 weeks	After 8 weeks
Kurtosis (Ideal value: 0)	Below left eye	0.49	0.44	0.42
	Left upper arm	0.97	0.40	0.35*
	Poll	0.82	0.55	0.29*
SEsm (Ideal value: Lowest value)	Below left eye	358.4	317.6	285.1*
	Left upper arm	344.6	364.2	319.0
	Poll	423.7	378.0	325.8*
SEr (Ideal value: Lowest value)	Below left eye	0.35	0.36	0.24
	Left upper arm	0.32	0.28	0.25
	Poll	0.45	0.46	0.42
SEsc (Ideal value: Lowest value)	Below left eye	246.4	177.0	123.8*
	Left upper arm	328.5	201.7	135.7*
	Poll	357.1	207.6	198.5
SEw (Ideal value: Lowest value)	Below left eye	40.2	29.0	22.3*
	Left upper arm	37.4	26.9	30.3
	Poll	28.4	25.7	27.1

Wiscoxon test: *:p<0.05

Table 8

		NAG group (n=10)		
		Before ingestion	After 4 weeks	After 8 weeks
Kurtosis (Ideal value: 0)	Below left eye	0.42	0.46	0.40
	Left upper arm	1.07	0.89	0.75
	Poll	0.81	0.73	0.68
SEsm (Ideal value: Lowest value)	Below left eye	311.7	319.7	289.2
	Left upper arm	324.4	318.5	320.6
	Poll	397.9	318.5	290.1*
SEr (Ideal value: Lowest value)	Below left eye	0.38	0.31	0.34
	Left upper arm	0.29	0.28	0.28
	Poll	0.60	0.56	0.52
SEsc (Ideal value: Lowest value)	Below left eye	262.1	270.4	208.3
	Left upper arm	356.9	291.2	235.7
	Poll	337.6	328.1	357.8
SEw (Ideal value: Lowest value)	Below left eye	38.3	32.7	32.3
	Left upper arm	38.4	36.5	35.2
	Poll	29.6	30.5	25.9

Wiscoxon test: *:$p<0.05$

Table 9

		C group (n=10)		
		Before ingestion	After 4 weeks	After 8 weeks
Kurtosis (Ideal value: 0)	Below left eye	0.40	0.41	0.42
	Left upper arm	1.15	1.09	0.92
	Poll	1.12	0.73	0.57*
SEsm (Ideal value: Lowest value)	Below left eye	392.1	333.4	349.1
	Left upper arm	344.9	331.4	329.8
	Poll	371.7	367.3	371.9
SEr (Ideal value: Lowest value)	Below left eye	0.40	0.38	0.39
	Left upper arm	0.28	0.28	0.26
	Poll	0.54	0.52	0.54
SEsc (Ideal value: Lowest value)	Below left eye	305.2	308.4	296.5
	Left upper arm	322.6	291.0	245.8
	Poll	347.6	330.6	325.6
SEw (Ideal value: Lowest value)	Below left eye	36.3	34.8	33.3
	Left upper arm	35.7	36.5	31.7
	Poll	30.7	30.1	29.9

Wiscoxon test: *:$p<0.05$

TEST EXAMPLE B (enzyme susceptibility of collagen peptide)

Preparation of Collagen Peptide

(Cod bone)

Twenty-four liters of water was added to 12 kg of a preliminarily decalcified cod bone and its adjacent portion to conduct extraction under heating (95.degree. C., 3 hours). After cooling, this extract was subjected to separation into solid and liquor with a wire net of 16 mesh, and then filtrated with a filter paper to obtain 30 liters of extract liquor containing collagen (Brix 6.0%).

This extract liquor was adjusted to pH 7.0 and heated to 60.degree. C. 3.6 g of a preparation of protease (tradename: "Protease N", manufactured by Amano Enzyme, hereinafter the same applies) was added thereto, and enzymatic reaction was carried out for 60 minutes. To this enzymatic reaction liquor, 360 g of activated carbon (tradename: "Taiko SW50", manufactured by Nimura Kagaku, hereinafter the same applies) was added. This liquor was heated to 80.degree. C. for 15 minutes, and then cooled, followed by filtration with a filter paper to obtain 25 liters of a filtrate (Brix 6.0%).

This filtrate was subjected to treatment with a membrane using a reverse osmosis membrane having a salt-preventing rate of 10% (tradename: "NTR-7410", manufactured by Nitto Denko Corp.), to obtain 11 liters of a concentrated liquor (Brix 12.0%). This

concentrated liquor was spray dried to obtain 900 g of a white collagen peptide powder.

With respect to this collagen peptide powder, free amino acid content, arsenic content, viscosity and number average molecular weight were measured by the following methods.

(1) Free amino acid content: HPLC method,

(2) Arsenic content: atomic absorption spectroscopy,

(3) Viscosity: a 10 wt % aqueous solution of a sample was prepared and then the viscosity at 20.degree.C. was neasured by a B-type rotation viscometer,

(4) Number average molecular weight: HPLC method

As results, (1) the free amino acid content was 0.6%, (2) the arsenic content was at most 2 ppm, (3) the viscosity was 24 cps and (4) the number average molecular weght was 2,400.

(Animal)

Commercially available collagen peptide prepared from animal was obtained. The chemical characteristic of the collagen peptide that was prepared from animal was comparable as in preparation from cod bone. With respect to the molecular weight of collagen peptide, it was measured by a conventional HPLC method and determined as an average 3,000.

Enzyme Susceptibility of Collagen Peptide

Test methods:

Collagen peptide, diluted to a concentration of 1.0 % (w/v), was subjected to an enzymatic digestion with pepsin in the buffer solution (pH 1.8), at 37.degree.C. for 24 hours. After 24-h reaction, the pH of mixture was adjusted to pH 8.2. An enzyme mixture of pancreatin and trypsin was added to the resulting mixture, and was continued to react with further for 24 hours at 37.degree.C.. After 24-h reaction, an additional enzyme of aminopeptidase was further added to the mixture to allow the reaction for 24 hours. An aliquot of solution at each step after the enzymatic digestion was collected and subjected to the NINHYDRIN test in order to analyze the amino acid content. The percentage of the total content of freed amino acids to the total content of amino acids, which were derived from in the starting collagen, was determined, and the increased portion of the value after individual reaction was considered as the contribution of the corresponding enzyme. The total amount of particular amino acids of interest, freed in the final sample solution after completion of enzymatic digestion, was also determined by using a conventional method with an amino acid analyzer. (L-8500A manufactured by HITACHI co.).

TEST RESULTS

As seen in Fig.1, by the representing column filled open, hatched and closed, which indicates a portion of contribution

by each indicated enzyme, it is clear that collagen peptide from cod bone is more susceptible to the enzymatic digestion, especially to an aminopeptidase.

As seen in Fig.2, by completion of enzymatic digestions, nutritionally essential amino acids liberated from fish collagen was more abundant than that derived from animal. Hydroxyproline (quantified as total amount with proline), which is known to be rich in collagen and one of active substances for human health, was also plentifully provided from fish collagen, as shown in Fig.3.

4. Consideration

From the above experimental results in test example A, it is found that the NAG + collagen ingestion group shows increase in moisture content of skin at a significant level and improvement in desiccation of skin, as compared with the NAG ingestion group and the collagen ingestion group. Further, from the results of dermatologic examination and doctors' questions and the results of analysis by microscopic three-dimensional skin surface, it is also found that the NAG + collagen ingestion group shows improvements in various symptoms of skin, as compared with the NAG ingestion group and the collagen ingestion group.

From the above experimental results in test example B, collagen peptide prepared from cod bone is more susceptible to the enzymatic digestion, especially to an aminopeptidase. In consistent with high susceptibility to the enzymatic digestion,

enhanced liberation of freed amino acids from fish collagen peptide was observed.

5. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements are the like so made are punishable by fine or imprisonment, or both, under Section 1001, of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 2004. 5. Apr

Yoshiharu MATAHIRA

Fig. 1

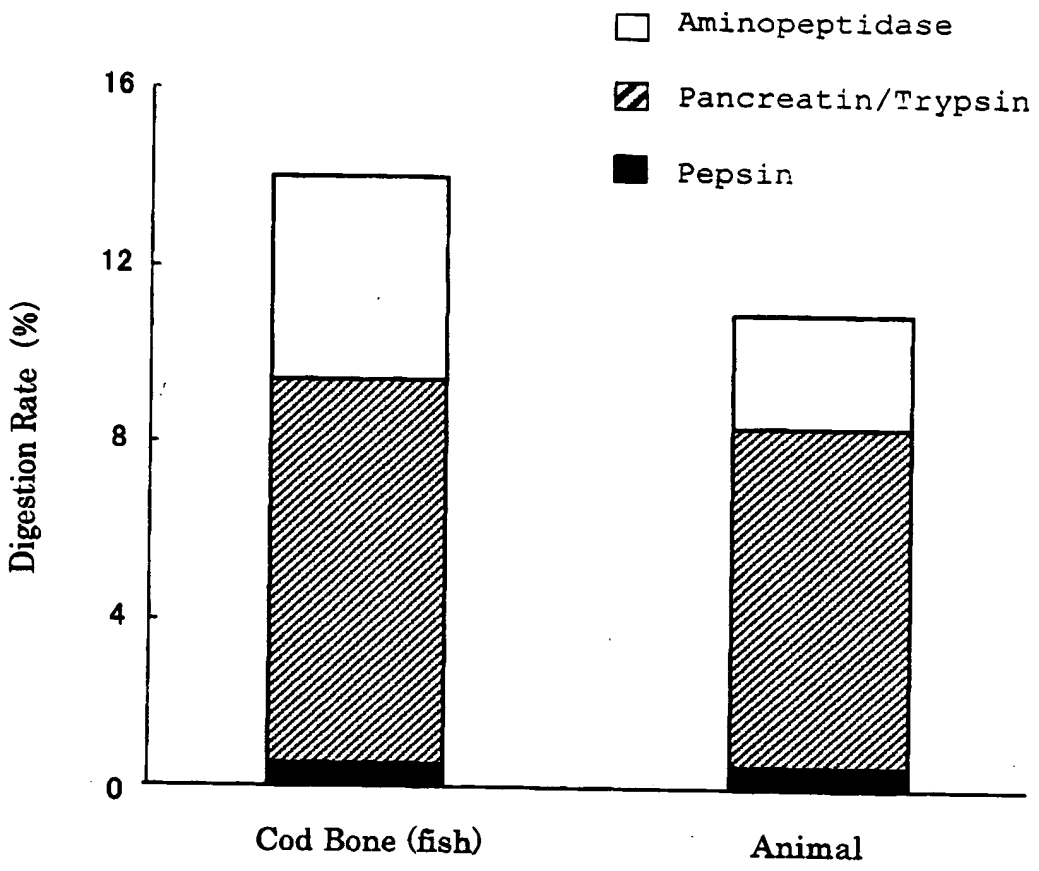

Fig. 1 The collagen peptide, prepared from the indicated biological source, was diluted to a concentration of 1.0 % (w/v), and subjected to an enzymatic digestion successively with pepsin , an mixture of pancreatin and trypsin, and aminopeptidase in order. The successive enzymatic digestion was performed for 24 hours at 37.degree.C in each step in a solution as details described in TEST EXAMPLE B. An aliquot of solution at each step after the enzymatic digestion was collected and subjected to the NINHYDRIN test in order to analyze the amino acid content. The percentage of freed amino acid to the total content of amino acid in the starting collagen peptide was determined. The increased portion of the value after individual reaction was considered as the contribution of the corresponding enzyme.

Fig. 2

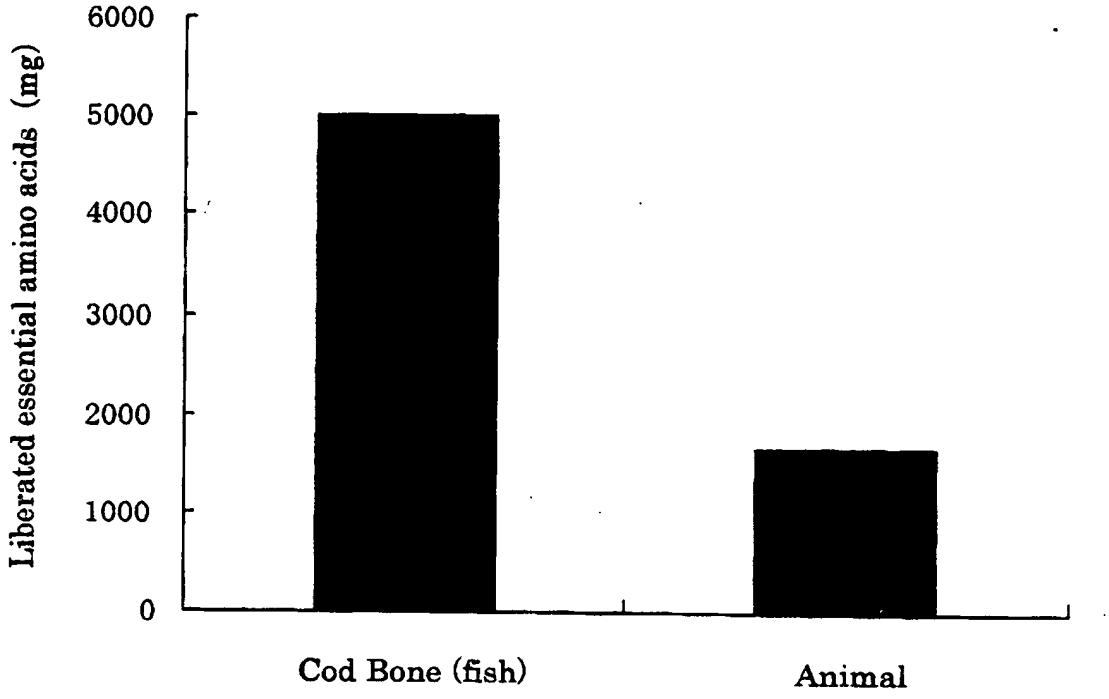

Fig. 2 The total amount of nutritionally essential amino acids liberated from the collagen peptides by completion of the successive digestion in Fig. 1 was determined by using a conventional method with an amino acid analyzer. (L-8500A manufactured by HITACHI co.)

Fig. 3

Fig. 3 The total amount of Hydroxyproline and Proline acids liberated from the collagen peptides by completion of the successive digestion in Fig. 1 was determined by using a conventional method with an amino acid analyzer. (L-8500A manufactured by HITACHI co.)